THE EFFECT OF TEMPERATURE ON THE INFECTIVITY AND PROPAGATION OF WESTERN EQUINE ENCEPHALITIS VIRUS

by

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INTRODUCTION

On the basis of contemporary epidemiological and epizoological information, the range of environmental temperatures that the arthropod viruses are exposed to is obviously considerable. Among the known natural hosts and vectors of the virus of Western equine encephalitis (WEE), a broad spectrum of normal body temperatures is evident. Under natural conditions of infection this virus is propagated at the elevated body temperatures of the human, horse and other mammals and at the low ambient temperatures of the significant vector of this virus, Culex tarsalis. Therefore, it would seem essential to a further understanding of the natural life history of the virus of Western equine encephalitis to study, under experimental conditions, the effects of temperature on certain of its biological properties.

Most of the experimental data available in the literature dealing with the effect of environmental temperature on host-virus interaction have been studied in vivo. Hyperpyrexia and hypopyrexia provide an environment that will select the members of the invading population that can best survive and multiply under the existing conditions. The most common host response to a viral disease is fever, therefore, by far the greatest amount of in vivo work has been concerned with elevated temperatures.

The use of cells cultivated in vitro provides an excellent means for study of the effect of temperature on the host cell-virus interaction. The cell culture system provides a greater number of controlled conditions under which experiments may be designed and carried out.

The studies presented in this thesis were undertaken to provide some information on the effect of propagating WEE virus at varied temperatures. Emphasis has been placed upon studies of the rate of propagation and cytopathogenicity of WEE virus as a function of temperature. It was felt that the results obtained may provide some basic information pertinent to the effects of environmental temperature on host-virus interactions. Modifications of classical experiments were used in this research which may provide a means of comparison with results obtained at other temperatures.

REVIEW OF THE LITERATURE

There is a wealth of literature pertaining to many aspects of the properties of Western equine encephalitis virus. Although the research presented in this thesis is primarily concerned with this virus, when relevant the literature reviewed considers other viruses as well.

A. HISTORY

Encephalitis has been observed in equine animals for many years. One of the casual agents (1) was isolated by Meyer in 1931 from the central nervous system tissues of an affected horse by inoculation into experimental animals. The isolated agent is now known as Western equine encephalitis virus. WEE virus was recovered from the CNS tissues and blood of man in 1938 by Howitt (2, 3) by intracerebral inoculation of mice. Since these original isolations from naturally infected horses and humans it has been demonstrated by immunological and isolation techniques that WEE virus infects a great variety of animals.

The most extensive epidemic of human encephalitis caused by WEE virus occurred in 1931. The area involved included North Dakota, Minnesota, and adjacent provinces of Canada where more than 3000 people contracted the disease. At the present time endemic foci exist over most areas of the Western United States and particularly along the Pacific Coast.

B. EPIDEMIOLOGY AND EPIZOOLOGY

The epidemiological and epizoological aspects of WEE virus have been studied by many workers. The review that follows will consider some of this work.

To study the method of spread of WEE virus in animal and human populations, both vectors and spread by contact were considered. Following the 1933 epidemic of encephalitis in St. Louis, it was reported that either mosquitoes were involved as vectors, or the infection was spread by contact. Attempts were made to try to transmit Eastern equine encephalitis (EEE) and WEE by mosquito bite. The results were positive; WEE virus was transmitted by seven types of Aedes mosquitoes (4). A study was conducted to determine if WEE and St. Louis encephalitis viruses could be isolated from mosquitoes present in an area where human cases of encephalitis were occurring or had occurred the previous year. WEE virus and St. Louis encephalitis virus were recovered only from C. tarsalis mosquitoes. (5).

During the year 1942 Hammon et al. (6) tested by animal inoculation 15,610 arthopods from the Yakima Valley of Washington for the presence of viruses. WEE and St. Louis encephalitis viruses were only recovered from C. tarsalis. Isolations were made from mosquitoes collected between July 9 and August 18 although collection actually started some two months previous to the first isolation and ended three weeks after the last isolation. The authors suggested that

the failure to isolate viruses earlier may have been due to the techniques employed.

After the virus was recovered in nature from C. tarsalis the next problem was to determine if C. tarsalis could transmit the disease under laboratory conditions (7). Laboratory reared C. tarsalis mosquitoes were allowed to feed on an experimentally infected guinea pig and duck. The mosquitoes transmitted the virus to chickens after an incubation period as short as ten days and for as long as thirty days.

Subsequent investigations have indicated that C. tarsalis is an efficient vector of transmission of American arthropod-borne encephalitis viruses (8). When given a blood meal containing a high concentration of any of the three viruses, nearly all of the mosquitoes become infected and 85 per cent subsequently become capable of transmitting the viruses by biting. It was found that a blood meal with a virus titer of 10 mice LD_{50}, would infect 1 to 5 per cent of the mosquitoes ingesting it and that up to 10,000 times as much virus could be recovered as had been ingested. Virus multiplication in mosquito tissues in vitro had been proven earlier by Trager (9).

In a study conducted in the Yakima Valley, Reeves and Hammon (10) found large numbers of adult C. tarsalis mosquitoes in domestic chicken houses, and concluded that the domestic chicken must be the preferred source of a blood meal for C. tarsalis. Through the use of agglutination tests it was found that C. tarsalis feeds on many different vertebrates

including the cow, horse, dog, chicken, human, pig and sheep. The possibility as to whether chicken immune serum agglutinated other bird blood was unknown (11). Reeves and Hammon (10) summarized that C. tarsalis, the species best fitting the epidemiological picture as a vector of equine encephalitis in the Yakima Valley, fed frequently on domestic and other fowl and included most of the common domestic animals and man in its feeding range. Mosquito species which were rarely or never found infected in nature, appeared to be those which fed almost exclusively on mammalian blood. In 1948 (12) it was observed that the blood found in many mosquitoes was not from chickens. Furthermore, it was demonstrated by use of an adsorbed specific antiserum, that the blood present in many mosquitoes was not of chicken but of other fowl origin. These findings demonstrated that wild fowl may play a significant role in the spread and maintenance of WEE virus.

In trying to determine the source of virus for mosquitoes, Hammon et al. (13) proposed that it was likely that mosquitoes become infected principly from animals which fulfill the following requirements: (a) They should be abundant, (b) they should show no apparent signs of infection, because no epizootic had ever been observed except in horses, and horses were relatively few in number, (c) they should have, as a result of a small peripheral inoculation, a reasonably large amount of virus circulating in the blood for more than a fleeting period of time, (d) they could theoretically be birds, because in an area where epidemics occur annually,

the reservoir animal should be one which does not bestow a first season's protection to its offspring by maternal transmission of antibody as frequently occurs in mammals.

In the summer of 1950 and 1951 in Weld county, Colorado, collections of sera from nestling wild birds were made to obtain evidence of the extent of infection of birds with WEE and St. Louis encephalitis virus. The sera were later tested for neutralizing antibodies against the two viruses. In the course of the survey it was discovered that some nestling birds, only one or two weeks old, had quite high neutralizing antibody titers against one or the other of the viruses. These findings were interpreted as meaning that these high titers were either the result of infection with the corresponding virus during the first few days after hatching, or that the antibodies had been acquired by the offspring by passive transfer from an immune mother by way of the egg (14).

Subsequent investigations to test the transovarian hypothesis revealed that a number of pigeon eggs collected in the study area contained a substance capable of neutralizing WEE and St. Louis encephalitis viruses (15). Some of the embryonic birds, and others immediately after hatching, possessed this neutralizing substance.

Reeves, et al. (16) using a dove colony found that seventy-five per cent of the offspring from parents experimentally infected with WEE virus had a neutralization index against WEE virus of 20 or greater. It was found that the index was highest in the birds hatching

during the first sixteen weeks after inoculation of the parents and that all offspring tested at sixteen weeks of age were completely lacking in the protective substance to WEE virus. Eight of the young birds were inoculated with WEE virus after loss of maternal antibodies. Three of these birds exhibited a viremia 24 to 48 hours later.

Fowl, wild and domestic, have been shown to be the principle source of WEE virus in nature (10, 13, 17) during the spring and summer months. Virus has been isolated from many species of birds (18, 19, 20).

The determination of the reservoir of WEE virus in nature has been a complex problem, with much emphasis being placed on the bird as the significant reservoir during the spring and summer months. WEE virus has been isolated from small mammals in nature. Lennette et al. (21) reported the recovery of WEE virus from the tree squirrel (Sciures guseus), and from the ground squirrel (Citellus beecheyi) in California. Isolations were made from animals collected from inside and outside a known endemic area. These authors concluded that the squirrel is highly susceptible to infection and not a true reservoir of WEE virus. During the survey made in Weld County, Colorado, sera from many species of small mammals were tested for the presence of neutralizing antibody, but almost all were found to be negative (14).

From the literature cited it has been demonstrated that avian hosts, wild and domestic, and animals provide the principle source of a blood meal and virus for C. tarsalis during the spring and summer months.

However, the reservoir (s) of the virus through the winter months has not been determined. One of the over-wintering mechanisms postulated has been a continuing bird-mosquito-bird cycle in warmer climates throughout the year, with seasonal invasion into more northern areas, through the intermediary of migrating birds (22). Blood from resident birds and birds migrating into Florida and Southern Louisiana was tested for the presence of WEE virus. Specimens failed to yield isolates of WEE virus.

Another over-wintering theory is that hibernating female C. tarsalis mosquitoes harbor the virus through the winter months. Blackmore and Winn (23) reported the isolation of WEE virus from hibernating C. tarsalis mosquitoes found in abandoned mine shafts. Pools were made containing 50 female C. tarsalis. The pool showing virus was one of 62 prepared from mosquitoes collected during December, January and February. Isolation was made using the "wet" chick method.

Experimentally infected C. tarsalis maintained at 55° F have been found to harbor WEE virus for 41 days (24). WEE virus survived up to 113 days in experimentally infected C. tarsalis maintained in the winter in an unheated cellar. The mosquitoes were able to transmit WEE virus to chickens after 97 and 109 days of winter incubation. Virus has been isolated from naturally infected C. tarsalis during all months of the year except December in Kern County, California (25). The peak incidence of WEE virus in C. tarsalis was from May to July. Strains isolated from

January through March had characteristics of attenuation or low titer, as they were non-pathogenic for mice and poorly immunogenic for chickens.

Attempts have been made to pass WEE virus through the eggs of the mosquito C. tarsalis (8, 26). Negative results were obtained from infected mated and unmated mosquitoes.

Mosquitoes other than C. tarsalis have been studied as possible vectors of WEE virus (4, 6, 7, 10). Other ectoparasites of birds also may be involved in the maintenance of WEE virus in nature. Mites have been considered as possible vectors of WEE virus and as vectors could preserve the bird-vector-bird cycle through the winter. Extensive studies have been done on this problem by Sulkin et al. (27) Reeves et al. (28) and Cockburn et al. (14). The conclusion reached was that mites do not act as vectors for WEE virus. Similar work was done by Winn and Bennington (29). English sparrows were inoculated with 140 chick LD_{50} amounts of WEE virus. Nasal mites were collected from the sparrows 20 days after injection. An attempt was made to isolate WEE virus from the mites using the "wet" chick method. No isolation of WEE virus was made.

The WEE virus may persist in avian hosts for as long as 10 months, and the virus may recirculate in the blood stream subsequent to the period of active viremia (30). Two-hundred eighty-four wild birds were kept in a mosquito proof outdoor aviary. Each bird was inoculated subcutaneously with WEE virus and their tissues and blood were tested for

virus one or more months after injection. Virus was isolated from blood, or organs of only eight of the birds. It should be emphasized that virus was isolated from eight of the 284 birds inoculated.

It has been suggested that garter snakes (Thamnophis species) may be possible reservoirs of WEE virus in nature and also a possible over-wintering host (31, 32, 33). C. tarsalis over-winters in rock piles and snakes also hibernated in these sites. Infected mosquitoes could pass the virus to snakes during the hibernation period or as both immerged from hibernation. Laboratory investigations have revealed that C. tarsalis feeds on garter snakes in the absence of other hosts (33). Experimentally infected C. tarsalis were allowed to feed on three garter snakes. Viremia of 4 to 20 days duration was observed. In one snake virus was detected in a 10^{-6} dilution of blood, which was the highest dilution tested. This quantity of virus in a host's blood has been reported to be sufficient to infect mosquitoes (8). Four snakes were also injected with a suspension containing 10^4 or 10^6 LD_{50} of WEE virus as determined by titration in suckling mice. Virus was detected in the blood of each snake. The titer of virus in one snake was at least 10^{-7}, the highest dilution tested. Virus persisted in one snake for thirty-six days at which time the snake died as did most of the snakes used in the experiments. There was no indication that WEE virus was responsible for their death (33).

WEE virus has been found in the blood of experimentally infected garter snakes 19, 64, 92, and 139 days after inoculation and their

hibernation in outside underground cages (31). The titer of virus in the blood of the infected snakes decreased during the coldest months of the winter. As the weather warmed the snakes immerged from hibernation and became active. An increase of virus titer in the blood was noted at this time. Similar results have been reported by others (32). An attempt was made to hibernate C. tarsalis in cages containing garter snakes. No mosquitoes were recovered following hibernation (32).

On the basis of the above observations, it has been suggested that garter snakes may be a possible over-wintering host of WEE virus (31, 32). The virus, however, has not been isolated from garter snakes collected in the field (32).

C. EFFECT OF TEMPERATURES ON THE INFECTION AND MULTIPLICATION OF VIRUSES.

The environmental temperature may affect the host-virus interaction basically in two ways: the effect of temperature on the host; the effect of the temperature on the virus.

Temperature may affect the host's defense mechanisms as low temperature has been shown to have a definite effect on antibody production (34, 35). Lwoff et al. (36) determined that keeping poliovirus infected mice at 37° C increased their resistance to the disease. It was suggested that hyperpyrexia may result in a latent infection. The resistance of rabbits against myxoma virus was decreased when the infected rabbits were kept at 10° to 23° C as compared to those maintained at 35° to 38° C (37). Adult mice kept in the cold (4° C) lost

their resistance to the Connecticut strain of coxsackie B1 virus (38). Environmental temperature has little effect on the mortality of mice infected with Influenza A virus (39). Mice maintained at a lower temperature show significantly more pulmonary involvement than those kept at a higher temperature. Rates of rabies infection in experimentally infected bats were higher when the bats were maintained at 29° C than at 5° or 10° C (40). The authors concluded that at the lower temperatures both virus and host were essentially inactive. WEE virus has been found to reduce in titer or disappear completely from the blood of experimentally infected garter snakes during the winter months (31, 32). As the weather warmed and the snakes became more active, the virus titer in the blood increased (31).

The effect of temperature on viruses may result in selection of the members of an invading population that can best survive and multiply under the existing conditions. For example there is a direct correlation between virulence and ability to propagate at a higher temperature with poliovirus (41). Poliovirus has been shown to have a decreased virulence for monkeys when passed at 23° C (42).

The effect of environmental temperature on virus infections in plants has been studied (43, 44) and a review published (45).

The preceding discussion presented some of the more important experimental data concerning the effect of temperature on the host-virus interaction in vivo. Another method of studying the host-virus

interaction is to use a cell culture system. Using this type of system characteristics of propagation, virus yield, adsorption and many other details of host-virus interaction may be studied. The cell culture system provides an excellent means for studying the effect of temperature on the host-virus interaction. In the following section some of the important data on the effect of temperature of incubation on the infectivity and production of virus in cell culture systems are presented.

To consider the effect of temperature on the interaction of a virus and selected host cells, the particular system must be first studied at 37° C and then compared with results obtained at various selected temperatures. Dulbecco and Vogt (46) studied the one-step growth curve of WEE virus at 37° C using chick embryo cells in suspension and maintained as monolayer cultures. The yield of virus from a single cell was also determined. The latent period lasted 2 1/2 hours at a multiplicity of 4 and 3 1/2 hours at 0.15. After the latent period the number of plaque forming units (PFU) increased in both cases, first exponentially for 1 1/2 hours, and then more slowly until a maximum was reached at 10 hours. The virus yield per cell was calculated by dividing the concentration of the virus at a given moment by the concentration of the virus-releasing cells as determined from the platings during the latent period. The maximum average yield of plaque-producing particles per cell varied between 100 and 200. Using cells maintained as a monolayer a small rise in

the curve was noted during the latent period. It was determined that the small rise in numbers of infective viruses was due to elution of a small fraction of the particles adsorbed. Upon the addition of 5×10^{-3} M KCN to the nutrient mixture, the first rise in the number of PFU per ml of the extracellular fluid was not suppressed, whereas the second rise was. The yield of virus particles per cell on a cell monolayer was calculated and found to be between 200 and 1000 PFU.

The one-step growth curve of WEE virus in L cells cultured as monolayers was investigated by Lockart and Groman (47). Compared to chick embryo cell cultures (46, 47, 48), virus production in L cells began later, showed a slower rate of increase and required a longer period of time to reach maximum titers. The average yield of virus based on maximum titers was from 200 to 700 PFU per L cell.

Poliovirus replication has been studied and was found to exhibit a typical one-step growth curve (41, 49). Second passage rhesus monkey kidney cells were grown for two days, infected with type I poliovirus (Mahoney strain) and then incubated at various temperatures between 4° C and 40.5° C (49). Although there was evidence of multiplication at all temperatures tested at or above 20° C it was found that the titer increased with increase in environmental temperature up to 39.5° C. Although the highest titer was obtained at 39.5° C, it was found that the release of virus at 37° C was more abundant in the initial phase of the growth curve. These investigations have shown that the environmental temperature has a very definite effect upon the replication of

this virus. It must be determined whether the temperature affects the actual production of the viral precursors within the host cell or some other stage in the process of virus synthesis such as adsorption of virus to the cell surface or release of the infective particle from the cell.

Adsorption studies of WEE virus by chick embryo cells were performed by Dulbecco and Vogt (46). To study the adsorption rate of WEE virus onto a monolayer of chick embryo cells, replicate cultures were infected with a constant amount of virus and incubated at 37° C. After various lengths of time samples were taken and the titer of each determined. The proportion of adsorbed virus increased rapidly with time up to 30 minutes. At the end of 30 minutes about 80 to 90 per cent of the virus had been adsorbed. After this time, the increase in adsorbed virus was very small. The rates of adsorption were found to be the same at room temperature and at 37° C (47). The attachment rate of poliovirus onto monkey kidney cells in the optimal attachment medium (10^{-3} M $CaCl_2$ in 0.25 M sucrose) has been determined (50). Temperature had no significant affect on the attachment rate. A slight decrease in the rate of attachment occurred with a lowering of temperature. This was interpreted as being due to an increase in the viscosity of the attachment medium.

The rate of intracellular appearance and release of WEE virus from chick embryo cells in suspension at 37° C has been studied (48). Intracellular and supernatant infective viruses were measured. The

data were used to determine the average time which elapsed between the moment a virus particle acquires the property of infectiousness within a cell and the moment it is released from that cell into the surrounding medium. This time was called the "release time" and was calculated and found to vary from 0.4 to 2 minutes, with an average of 0.9 minute. This finding indicates that WEE virus is released very rapidly after it gains the property of infectiousness.

In a quantitative study on the formation and release of herpes simplex virus (HSV) in FL cells at different temperatures of incubation (51) it was found that the highest rates of formation as well as the highest amount of virus were produced at 37° C. But it was also found that the most rapid rates of release, the largest quantities of infectious virus recovered per cell from the fluid phase, and the highest amounts of released virus relative to maximum intracellular levels, occurred at 37° C. The rate of HSV spread from cell to cell as a function of temperature has been studied by the microplaque technique (52). The size of the microplaque produced when FL cells infected with HSV were incubated at various temperatures (31°, 34°, and 37° C) was taken as the measure of the rate of spread of HSV virus from cell to cell. The temperature of maximum spread was 37° C. It is tempting to relate the rate of virus spread from cell to cell to the release of virus. Hoggan et al. (52) stated that several experiments indicated that this correlation may not be based on a causal relationship between virus release and cell to cell spread.

In the literature cited thus far it has been shown that the temperature of incubation has a definite effect upon the growth of a virus, that it has little or no effect upon the adsorption of the virus to the host cell, and that it effects the rate of release of virus from the infected host cell.

The effect of environmental temperature on the quantitative production of virus is dependent upon the particular virus being studied. The amount of poliovirus produced increases with an increase in environmental temperature up to 39.5° C (49). Some viruses, however, show maximum growth at lower temperatures. HSV produces the greatest quantity of virus at 34° and 35° C (51, 53). A higher titer, as determined by titration in mice, was obtained in chick embryo mince infected with WEE virus and incubated at room temperature rather than at 37° C (54). Similar results were obtained in serum-ultrafiltrate mouse brain cultures infected with St. Louis encephalitis virus (55).

If a virus can multiply at a particular temperature of incubation then the effect of the temperature of incubation on the virulence of the virus must be determined. Cold-adapted genetic variants of the poliovirus strains Akron (type I), Brooks (type II), and Mabie (type III) have been obtained by passage at 30° C in monkey kidney cells (56). The 30° C passed viruses, in comparison with the original viruses, had more rapid cytopathogenic action on monkey kidney cells in tubes incubated at 30° C. All three variants were deadapted to propagation at 36° C in monkey kidney cell cultures, but the deadaption of Mabie was much greater than that of Akron or Brooks. The authors considered the

possibility that the cold-adapted variants may be generally deadapted to propagation at temperatures as high as 36° C and, hence, may possess less virulence for a primate whose body temperature is somewhat higher than 36° C. The cold-adapted variants were compared with the original virus in virulence for cynomolgus and rhesus monkeys by the intraspinal route of inoculation. The data obtained showed that the cold-adapted variants of Brooks and Akron did not differ in virulence from their progenitors. The cold-adapted Mabie, however, was found to be less virulent than the original. Thus the strain that was strikingly deadapted to propagation at 36° C in cell culture was also the one that had become less virulent for the monkey. These results suggest that the degree of loss of ability of 30° C passaged virus to propagate at 36° C in cell cultures may serve as an indicator of the degree of their loss of virulence for warm blooded animals.

Lwoff (41) has attempted to correlate virulence of poliovirus with the ability of the virus to multiply at a high temperature of incubation. Three strains of poliovirus type I were studied. One was the highly attenuated strain used by Sabin as a live vaccine for immunization of humans. It was completely devoid of virulence for the monkey. The second was the Burnhilde-Enders strain which is of relatively low virulence for the monkey. The third was the highly virulent Mahoney strain. The data showed that the sensitivity of virus propagation to temperature differs with the strain. The more neurovirulent the strain, the less sensitive was its growth to high temperature effects. Several

other poliovirus strains were studied and the same correlation could be made. Further correlation of virulence of poliovirus in relation to variant characteristics in vitro was studied (42). Through the use of specific selective environments, several variant viruses were obtained which differed in in vitro characteristics. None of the variant polioviruses showed a reduced virulence for monkeys.

The literature cited demonstrates that some viruses propagated at a low temperature will be produced at a reduced rate of synthesis in infected cells. The release rate of the virus is reduced, and the adsorption of the virus onto the cell surface is independent of the temperature of incubation. Some viruses propagated at a low temperature have a reduced virulence for animals. The propagation of a virus at an increased temperature will also have a specific effect upon the virus involved. From these facts it can be seen that the temperature of incubation is a controlling factor in the propagation of a virus in cell culture. The extent of influence the temperature of incubation plays on host-virus interaction will depend upon the virus and cell culture system used.

D. STABILITY OF WEE VIRUS

The stability of a virus becomes an important factor when the experiment extends over several days. The maximum stability of WEE virus at 4° C is between pH 6.5 and 8.5. The stability of WEE virus drops off rapidly in suspensions more acid than pH 6.5. It can be

preserved in cold 50 per cent buffered glycerol, by being held in the frozen state, and by lyophilization (1).

WEE virus has been considered to be relatively unstable. Lockart and Groman (47) have studied the stability of WEE virus. WEE virus is inactivated rapidly at 37° C in fluids devoid of cells. The half life in a medium containing 80 per cent Earles' saline, 10 per cent horse serum, and 10 per cent chicken embryo extract was 4 hours. The half life in PBS was 17 minutes with about 90 per cent inactivation in 1 hour. PBS was permitted to remain in contact with chick embryo monolayers for 1 hour at room temperature. The PBS was then removed from the monolayers and its effect on virus inactivation determined over a 1 hour period. Only 3 to 20 per cent of the virus was inactivated in 60 minutes. A possible explanation for the reduction in virus inactivation in PBS which had been in contact with cells was that cysteine or other reducing substances were released from the cells. Similar results have been observed with Eastern equine encephalomyelitis virus at 37° C (57).

E. CYTOPATHOGENICITY OF VIRUS INFECTED CELL CULTURE MONOLAYERS

When many viruses multiply they produce degenerative changes in cell cultures which can easily be seen (58). This phenomenon was described by several workers (59, 60, 61). Its significance was not appreciated until about 1950. At this time it was emphasized that the changes produced in cultured cells by a virus was an adequate criterion

for demonstrating the presence of a virus (62). Furthermore it was demonstrated that the addition of specific antibody to the cell culture along with the virus inoculum prevented the development of cytopathogenicity.

Secondary cultures of rhesus monkey kidney cells infected with Type I poliovirus (Mahoney strain) do not develop cytopathogenicity when incubated at 40.5° C. However, the cells were found to be capable of growth and to exhibit cytopathogenicity when they were incubated later at 37° C (49).

WEE virus has been reported to persist for long periods of time in roller tube cultures of chick embryo cells (63) and stationary cultures of strain L cells (64) with little obvious cytopathic change in the cells. The strain L cells initially showed cytopathogenicity but a few of the surviving cells proliferated and completely replaced the lost cells.

MATERIALS AND METHODS

A. SOLUTIONS AND MEDIA

1. Growth Fluid for Chick Embryo Cell Cultures

The cell culture medium used in these studies was composed of Earles' balanced salt solution, 0.4 per cent lactalbumin hydrolysate, (Nutritional Biochemical Company), 4 per cent inactivated calf serum, 100 units per ml of penicillin and 100 ugm per ml of streptomycin. The medium was sterilized by filtration through a Seitz filter and allowed to stand at room temperature overnight to determine sterility and to equilibrate the pH. The final pH of the cell culture medium was approximately 7.2.

2. Earles' Balanced Salt Solution

Earles' balanced salt solution was prepared in 10X concentration and stored for short periods of time at 4° C unsterilized. The formula for this balanced salt solution is as follows:

NaCl	6.8 g
KCl	0.4 g
$CaCl_2$	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$NaH_2PO_4 \cdot H_2O$	0.14 g
Glucose	1.0 g
Phenol Red	0.02 g
Distilled Water	1000 ml

The $CaCl_2$ was added last with constant stirring to avoid precipitation. The 10X solution may be sterilized by autoclaving by leaving out the $CaCl_2$. The $CaCl_2$ was dissolved in distilled water, sterilized by autoclaving and added to the 10X solution after both solutions were cooled.

3. Phosphate Buffered Saline

Phosphate buffered saline (PBS) was prepared using the formula of Dulbecco and Vogt (65) which follows:

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
$CaCl_2$	0.1 g
$MgCl_2 \cdot 6H_2O$	0.1 g
Distilled water	1000 ml

By omitting the $CaCl_2$ this solution was made up in 10X concentration. The $CaCl_2$ was made up as a separate solution so that 1 ml added to 1 liter of 1 X PBS was equivalent to 0.1 g per liter. PBS was sterilized by filtration through a Seitz filter.

Magnesium and calcium deficient saline solution (PD) is PBS with the $CaCl_2$ and $MgCl_2$ omitted.

4. Trypsin Solution

Trypsin (Difco 1:250) solution was prepared according to the method of Younger (66) in PD at a concentration of 0.25 per cent and

sterilized by filtration through a Seitz filter. Trypsin solutions were discarded after three days if not used.

B. VIRUS STRAINS

The viruses of Western equine encephalitis (WEE) used in this research were obtained from the Virus Research Laboratory, Department of Microbiology, University of Utah. The original parent virus was isolated by inoculation of one-half-day-old "wet" chicks with tissues obtained from a fatal human case. The virus was passed several times through mice and the infected brain suspensions were used to inoculate garter snake embryo cell cultures maintained at 25° C. The fluid from the garter snake embryo cell cultures was used to infect primary chick embryo cell cultures. The virus was passed through chick embryo cell cultures ten times and then titrated by the plaque assay method of Dulbecco and Vogt (46). Progeny virus from a single plaque was used to propagate a stock pool of virus. With subsequent titration of the pool two distinct plaques differing in size were observed. Progeny viruses from different sized plaques were passed through chick embryo cell cultures and recloned at limiting dilutions. One variant, designated S. P. # 6, was isolated which produced plaques with an average diameter of approximately 2 mm. The other, designated L. P. # 7, produced plaques having a diameter of approximately 8 mm. Each variant was found to retain its plaque size character when passed through chick embryo cell cultures and suckling mice. Specific WEE

virus antisera obtained from the Communicable Disease Center was used to establish the identity of the two variants as WEE virus. FIGURE #1 and 2 are photographs which illustrate the morphology of the plaques produced by these variants on primary chick embryo cell monolayers. The photographs were taken 54 hours after infection of the cell monolayers (67).

C. CHICK EMBRYO CELL CULTURES

The method used essentially follows the procedure of Rubin et al. (48) and Welsh et al. (68) with modifications to simplify the procedures. Nine to ten day old chick embryos were removed aseptically from the eggs and placed in a sterile petri dish containing Earles' balanced salt solution or PD. The head, feet and wings were removed and the embryos minced with a pair of sterile scissors or two sterile scalpels. The tissues were transferred to a trypsinizing flask and washed three times on a magnetic stirrer with PD to remove undesirable material. The washed mince was treated with fresh 0.25 per cent pre-warmed trypsin in a trypsinizing flask on a magnetic stirrer four times for five minutes per treatment. After each treatment the mixture was allowed to stand for one minute to permit large particles to settle. The fluid containing the free cells was decanted through sterile gauze into a convenient sized flask which has been placed in an ice bath. Twenty-five ml of heat inactivated calf serum were added to each liter of trypsin-cell suspension. The cell-trypsin mixture was centrifuged for five to ten minutes at 600 to 800 RPM in an International

FIGURE # 1. Plaque formation by WEE virus variant S. P. # 6.

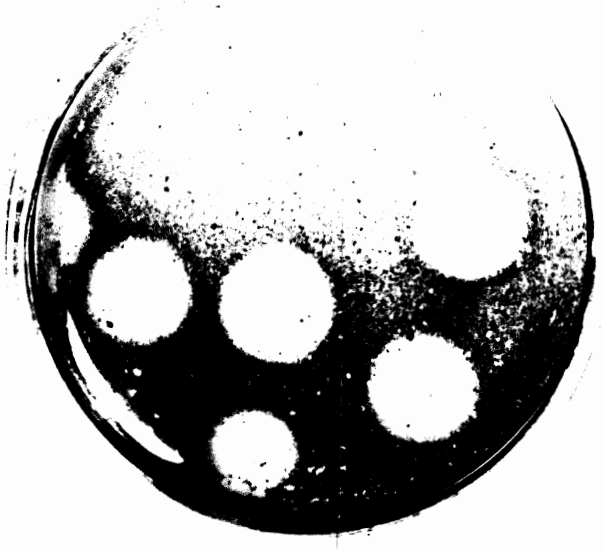

FIGURE # 2. Plaque formation by WEE virus variant L. P. # 7.

centrifuge (Model 1-S B). The supernatant was decanted and the cell pack resuspended in a small known volume of growth medium. A cell count was made using a hemocytometer and the cells were diluted to the desired concentration and distributed into cell culture vessels. Approximately 1 X 10^7 cells were inoculated into 60 mm glass or plastic petri dishes (Pyrex glass and/or Falcon plastic tissue culture dishes). Bottle cultures contained a greater or lesser number of cells depending upon the size of the bottle. Cell cultures in plates were incubated in a cell culture chamber (69) at 37° C under CO_2 tension. All chick embryo cell cultures used were primary cultures.

All fertile eggs used in this research came from the same line of chickens and were obtained from a local hatchery.

All cell counts were carried out using a hemocytometer. When necessary, cell dilutions were made in growth medium. In no case was it necessary to make a cell dilution of more than 1:100.

D. VIRUS TITRATION BY THE PLAQUE ASSAY METHOD

Virus titrations were carried out following the method of Dulbecco and Vogt (46) with some modifications. Chick embryo cell cultures were grown in 60 mm petri dishes at 37° C for 48 hours. For virus titrations by plaque formation a confluent monolayer is necessary; therefore, the resulting monolayers were checked microscopically for confluency prior to use. The growth fluid was removed and the cells infected by addition of 0.2 ml of the appropriate virus dilution which has been prepared in growth fluid. After an

adsorption period of 45 to 60 minutes at 25° C, the infected monolayers were covered with 4 ml of agar over-lay medium containing equal portions of double-strength growth medium and 1.8 per cent purified agar in distilled water. After twenty-four hours of incubation at 37° C, 1.5 ml of a 1:5000 neutral red solution were added. The cultures were incubated overnight and the plaques counted the following morning. The plaques appeared as clear areas in a red background.

Agar (Difco) was purified by repeated washings with distilled water, acetone and ether. A similar method was described by Dulbecco and Vogt (65).

E. INFECTIVE CENTER ASSAY

To determine the number of infected cells in a system an infective center assay was carried out. After the infection of the monolayer with virus and washing the infected cells three times with cold PBS, the cells were removed from the glass by trypsinization with a 0.05 per cent pre-warmed trypsin solution. Nine volumes of growth medium were added and the clumps of cells dispersed by rapid pipetting. The resulting cell suspensions were counted, diluted in growth medium, if necessary, and 0.2 ml of the desired dilution was added to a 60 mm petri dish containing a confluent chick embryo cell culture monolayer. Attachment of the cells was allowed to proceed for 1 hour at 37° C. After this time period, 1 ml of agar overlay medium was carefully added to each plate. After the medium was allowed to solidify an additional

3 ml of medium was added to each plate. Incubation and addition of neutral red solution was carried out as described previously.

EXPERIMENTAL RESULTS

A. PROPAGATION OF WEE VIRUS AT 25° AND 37° C

1. CELLS MAINTAINED AS A MONOLAYER

a. Experimental

The propagation of WEE virus variants L. P. # 7 and S. P. # 6 were studied at 25° and 37° C using chick embryo cell cultures. The cell cultures used were maintained as a monolayer and in suspension. Equal numbers of chick embryo cells were grown in 16 fluid ounce "prescription" bottles for 48 hours at 37° C. An approximation of the number of cells in each bottle was determined by counting the cells from replicate bottles. Cells growing as monolayers were infected with the two variants of WEE virus, L. P. # 7 and S. P. # 6. The cells were infected with a multiplicity of 10 and attachment was allowed to proceed at 25° C for 1 hour. The infected monolayers were washed two times with cold growth medium and 50 ml of 25° C growth medium were added to each culture. The zero time sample was taken immediately and the cultures placed in a 25° C incubator. Further 0.5 ml samples were taken at the specific time intervals indicated. Each sample was diluted 1:10 in cold growth medium, divided into three aliquots and stored at -20° C until assayed for infective virus. Each 0.5 ml sample removed was replaced by the addition of a similar amount of culture medium in order to maintain a constant volume in each bottle.

FIGURE # 3 presents the results of the data obtained at 25° C. The same number of PFU of the large plaque variant and S. P. # 6 were used to infect the monolayers. However, on titration of the zero time samples of the two plaque variants and calculation of the number of infective particles in that sample, it was noted that there was a higher number of detectable particles of S. P. # 6 than L. P. # 7. A partial explanation may be the elution of larger numbers of S. P. # 6 after washing of the infected cells. This relationship was exhibited through this study with one exception which is demonstration in FIGURE # 4.

The data showed a lag period of 10 hours for both strains of virus. By 12 hours after incubation at 25° C a detectable increase of infective virus in the extracellular fluid was noted. With both viruses the number PFU increased rapidly for 20 hours and then more slowly until a maximum was reached at 72 hours. After the maximum numbers of PFU were obtained the titers declined slowly till the end of the experiment of 144 hours.

The number of detectable PFU of S. P. # 6 increased at a slower rate and the titer obtained was not as high as was L. P. # 7. After 72 hours the decline in titer of S. P. # 6 was slower than L. P. # 7 and the titers at 144 hours for both were the same. On the basis of cell counts of replicate noninfected monolayers an estimate of the maximum number of PFU released per cell was determined. Cells infected with large plaque variant released a maximum of about 150 PFU per cell while cells infected with S. P. # 6 released 75 PFU per cell.

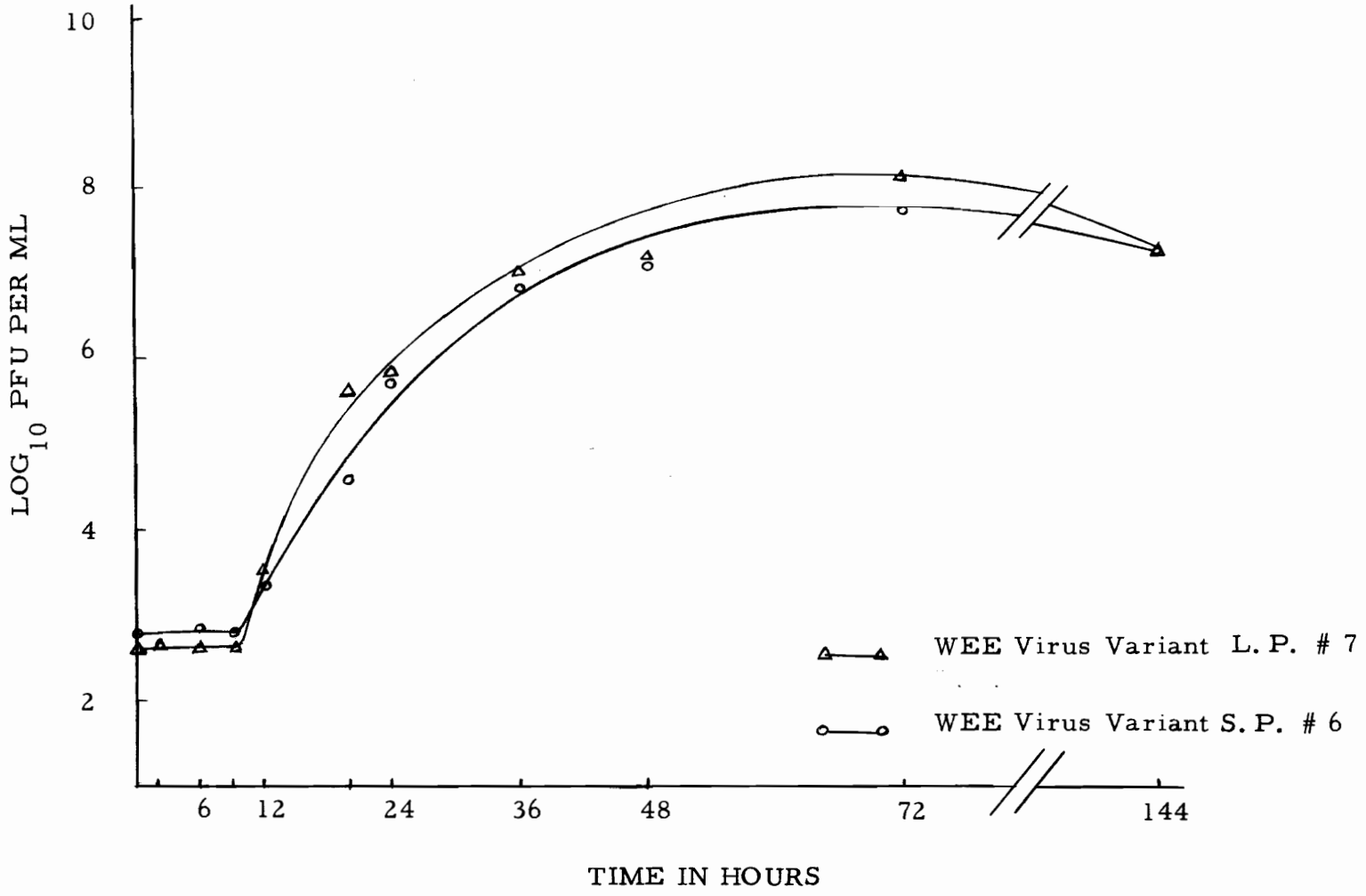

FIGURE # 3. Growth curves of WEE virus variants with cells maintained as a monolayer and incubated at 25° C.

To determine the effect of temperature on the propagation of the two variants, L. P. # 7 and S. P. # 6, the experimental procedures used were those previously described except that the variable, temperature, was 37° C. The results obtained are presented in FIGURE # 4. The zero time plaque count represents free extracellular virus. Plaque variant L. P. # 7 exhibited a lag period of 1 1/2 hours. The number of PFU released increased rapidly for 2 1/2 hours then more slowly until a maximum titer was reached at 8 to 10 hours.

Two hours after incubation of S. P. # 6 infected chick embryo monolayers a increase in PFU in the extracellular fluid was noted. The data shows that the PFU increased slowly for 1 hour and then exponentially for 3 hours. The maximum titer was obtained after 10 hours of incubation.

The initial plaque count of L. P. # 7 was more than that obtained for S. P. # 6. This observation was in direct contrast to the data obtained at 25° C. It was also noted that the number of detectable particles of L. P. # 7 in the extracellular fluid increased in number at a more rapid rate and the large plaque variant had a shorter lag period than did the small plaque variant. The number of PFU released per cell infected with L. P. # 7 and incubated at 37° C was about 2000. Cells infected with variant S. P. # 6 released about 100 PFU per cell.

In comparing the results obtained at 25° C with those at 37° C it can be seen that the lag period was longer, the increase in numbers

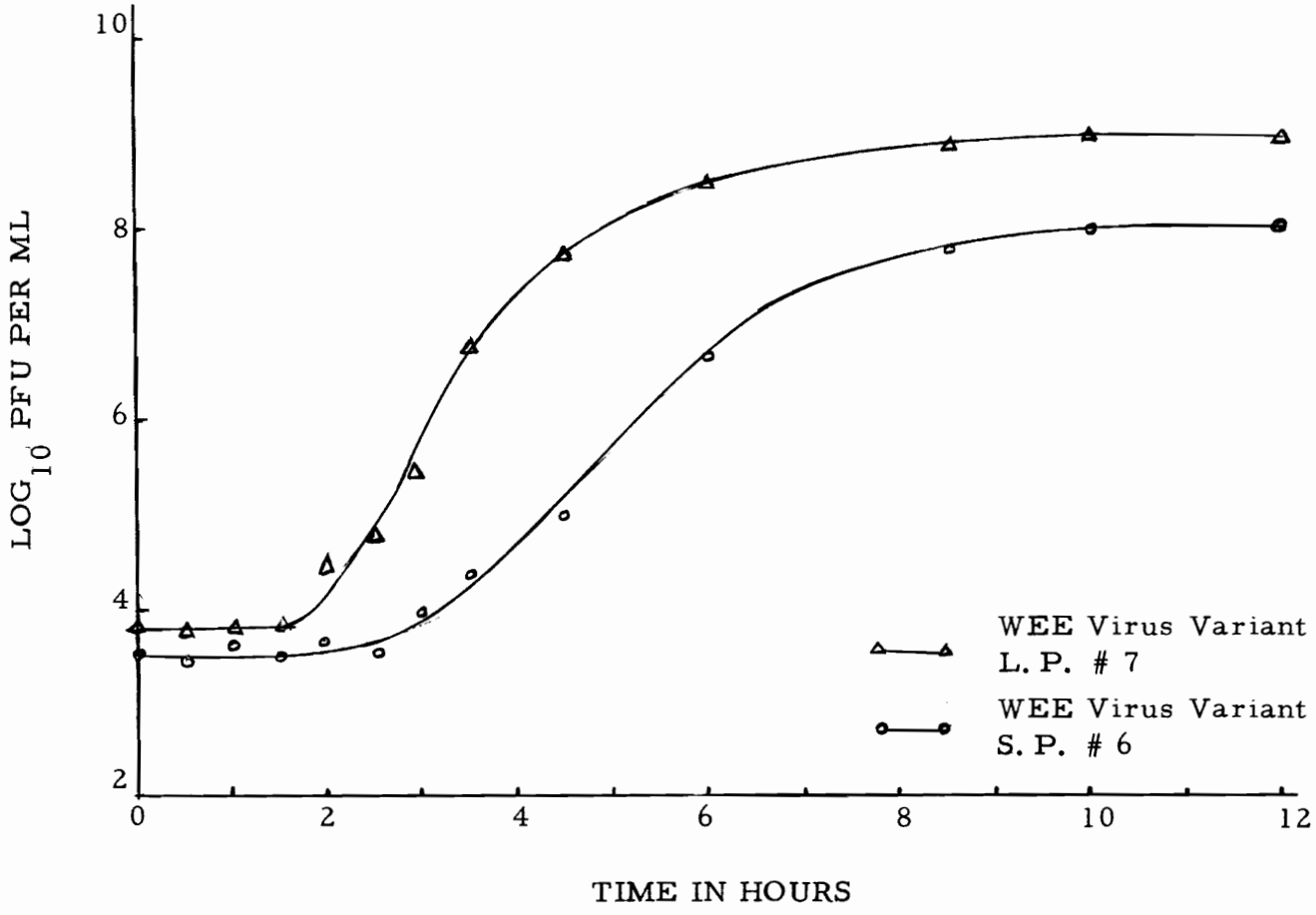

FIGURE # 4. Growth curve of S. P. # 6 and L. P. # 7 strains of WEE virus on chick embryo monolayer cell culture at a multiplicity of 10 and incubated at 37° C.

of PFU was slower, the maximum titer was less, and that the maximum number of PFU released per cell was also less.

2. CELLS IN SUSPENSION

a. Experimental.

Equal numbers of chick embryo cells were grown in 16 fluid ounce "prescription" bottles and infected with equal numbers of L. P. # 7 and S. P. # 6 at a multiplicity of 10. The number of cells in each bottle was determined by counting the cells from replicate bottles. Attachment was allowed to proceed for 1 hour at 25° C. After the attachment period the infected cells were washed three times with cold PBS. The washed infected cells were removed from the glass with pre-warmed 0.05 per cent trypsin and nine volumes of cold growth fluid were added to the infected cell-trypsin suspension. Cell clumps were dispersed by rapid pipetting of the suspensions. The cells were counted, diluted and a total of 3.5×10^5 cells were added to spinner flasks (Bellco) and the volume increased to 200 ml by the addition of 25° C growth fluid. The infected cells were maintained at 25° C in a constant temperature water bath with constant stirring. A zero time sample was taken for infective center assays and for determination of free virus present in the extracellular fluid. Samples were removed at the indicated times and cells removed by centrifugation. The supernatant fluid was removed and stored at -20° C for assay of infective virus. Identical experiments were conducted using the procedures as described with the infected chick embryo cells in suspension and incubated at 37° C.

The results obtained from the study of growth rates of variants L. P. # 7 and S. P. # 6 at 25° C are presented in FIGURE # 5. The lag period for both strains was 10 hours and the first increase in PFU was noted at 12 hours. The numbers of PFU of L. P. # 7 increased in number rapidly for 14 hours then slowly until a maximum was reached after 36 hours of incubation. The plaque count of the small plaque variant increased at a slower rate than did L. P. # 7 and also attained a higher titer.

On the basis of infective center assays, cells infected with L. P. # 7 released about 55 PFU per infected cell. The small plaque variant, however, released approximately 170 PFU per infected cell. These results were in direct contrast to those obtained with cells infected with the two plaque variants and maintained as a monolayer. In comparing the yield of PFU released per cell as a function of time it was found that L. P. # 7 released virus more rapidly than S. P. # 6. However, after 30 hours of incubation the data indicated that cells infected with L. P. # 7 did not release large quantities of virus after 36 hours of incubation. The yield of PFU per cell infected with S. P. # 6 continued to rise for 18 hours and then declined at the end of the experiment.

FIGURE # 6 presents the data obtained when WEE virus infected cells in suspension were incubated at 37° C. The length of the lag period of the two strains was the same as when the infected cells

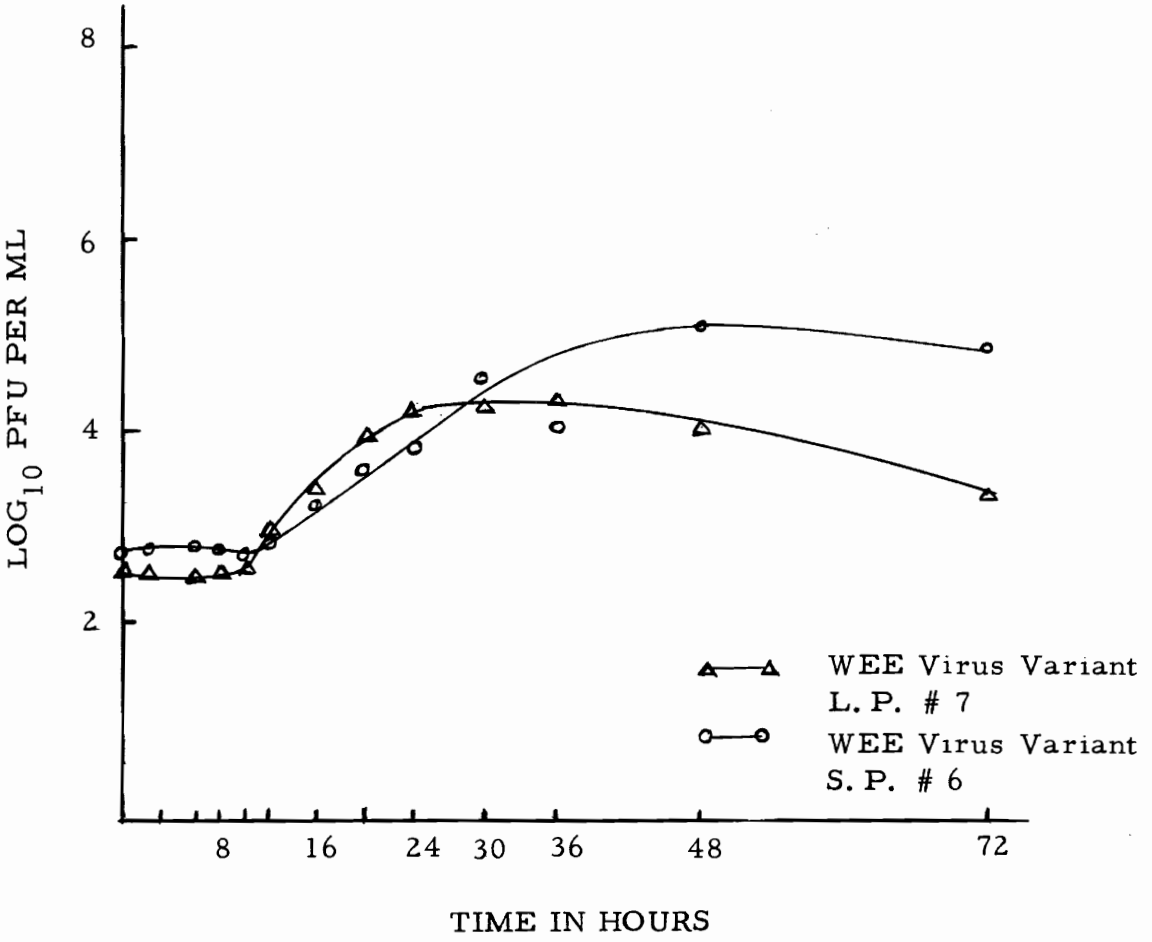

FIGURE # 5. Growth curves of plaque variants on suspended chick embryo cells incubated at 25° C.

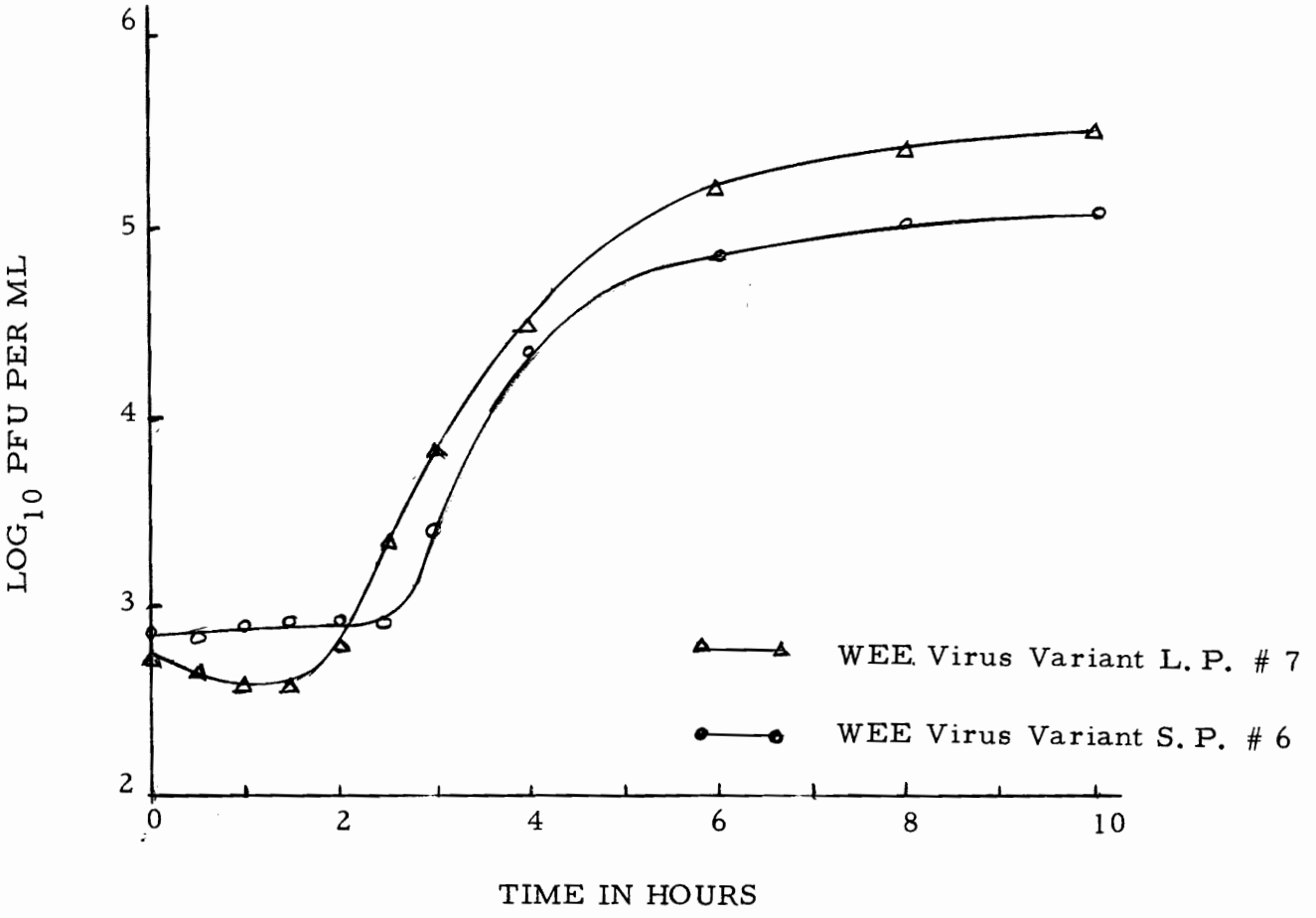

FIGURE # 6, Growth Curve of S. P. # 6 and L.P. # 7 Strains of WEE Virus on Suspended Chick Embryo Cells at a Multiplicity of 10 and Incubated at 37° C.

were maintained as monolayer cultures. L. P. # 7 had a lag period of 1 1/2 hours while the lag period for S. P. # 6 was 2 hours. The number of infective particles of L. P. # 7 increased exponentially for approximately 2 1/2 hours after the lag period. S. P. # 6 increased at a similar rate but for a shorter period of time. Both viruses reached a maximum titer by 10 hours. L. P. # 7, however, attained a higher titer than did S. P. # 6. As observed before the zero time plaque count of S. P. # 6 was greater than L. P. # 7 even though the two viruses were studied under the same conditions. The maximum yield per cell was calculated on the basis of infective center assay. Cells infected with the large plaque variant produced 1000 PFU per infected cell and S. P. # 6 infected cells released a maximum of approximately 100 PFU.

B. CELL CYTOPATHOGENICITY

1. WEE VIRUS INFECTED CELLS INCUBATED AT 25° C

a. Experimental.

During the study of the propagation of WEE virus at 25° C it was observed that cytopathogenicity was difficult to detect in monolayers of chick embryo cells infected with WEE virus. The control cells appeared to develop cytopathic changes after 48 hours of incubation at 25° C. An experiment was designed to study the changes that develop in infected and noninfected chick embryo cells incubated at 25° and 37° C.

Washed sterile cover glasses (Corning # 1, 22 X 22 mm) were placed in sterile 60 mm petri dishes. A total of 5 X 10^6 chick embryo

cells in a volume of 5 ml were added to each plate. The plates containing the cover glasses and cells were incubated under CO_2 tension at 37° C as previously described. At the end of the incubation period the growth fluid was removed from the plates and the monolayers were washed once with growth fluid. The cells were infected with WEE virus and attachment was allowed to proceed for 45 minutes at 25° C. Half of the infected and half of the noninfected monolayers were incubated at 37° C and the other half of the monolayers were incubated at 25° C. At specific time intervals infected and noninfected cell cultures were taken from the incubators, the cover glasses removed from the plates and the adherent cells fixed. The fixative consisted of 6 parts 95 per cent ethyl alcohol, 3 parts glacial acetic acid and 1 part neutral formaldehyde. After fixation, the cells were washed carefully in distilled water and air dried. The cells were stained with May-Grünwald Giemsa stain and the cover glasses mounted on glass slides with Permount (Fisher Scientific Company). The cells were studied for cytopathogenicity with the aid of a microscope.

Cells infected with WEE virus plaque variants L. P. # 7 and S. P. # 6 and incubated at 25° C did not develop any visable signs of cytopathogenicity until after 24 hours of incubation. FIGURE # 7, 8 and 9 are photomicrographs of noninfected and infected cells 8 hours after incubation at 25° C. The cells were fibroblastic in appearance with extensive vacuolation visible in the cytoplasm. The nucleus appears

as a distinct structure and the nuclear membrane can be observed. The nucleus stained a darker blue than the cytoplasm and hence is more basophilic than the cytoplasm. The cytoplasm of the cells was not retracted and cellular debris was not evident. No distinguishable characteristics could be observed between the infected and noninfected cultures at this time.

After 24 hours of incubation at 25° C the infected cells showed cytopathic changes as illustrated in FIGURES # 11 and 12. The most easily observed change was the retraction of the cytoplasm and cellular debris could also be seen. The nucleus remained normal in appearance. The 24 hour control cells (FIGURE # 10) appeared normal with no evidence of contraction of the cytoplasm or the presence of cellular debris. The degenative changes in the cells infected with S. P. # 6 were not as evident as the changes in cells infected with the large plaque variant. It was noted that cytopathogenicity developed at 25° C during the logarithmic phase of the growth curve, FIGURES # 1 and 3.

FIGURE # 13 demonstrates the morphology of noninfected chick embryo cells 72 hours after incubation at 25° C. The cells have remained fibroblastic in appearance and have decreased in number. The cytoplasm was more retracted than at earlier periods and there was a decrease in vacuolation. Cellular debris could be observed.

Cells infected with S. P. # 6 and incubated for 72 hours at 25° C are illustrated in FIGURE # 14. Cytopathogenicity was evident

FIGURE # 7. Noninfected chick embryo cells after 8 hours of incubation at 25° C.

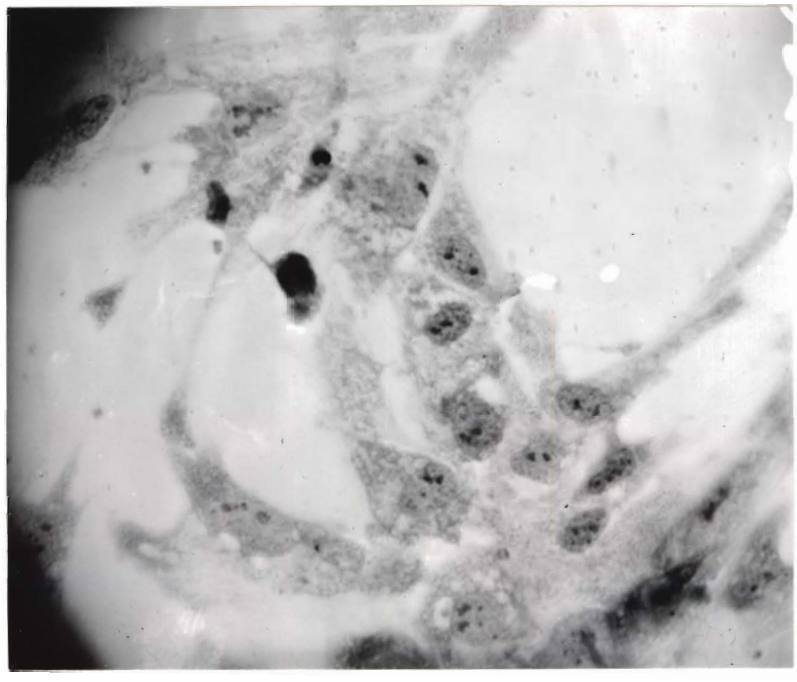

FIGURE # 8. Appearance of S. P. # 6 infected check embryo cells after incubation at 25° C for 8 hours.

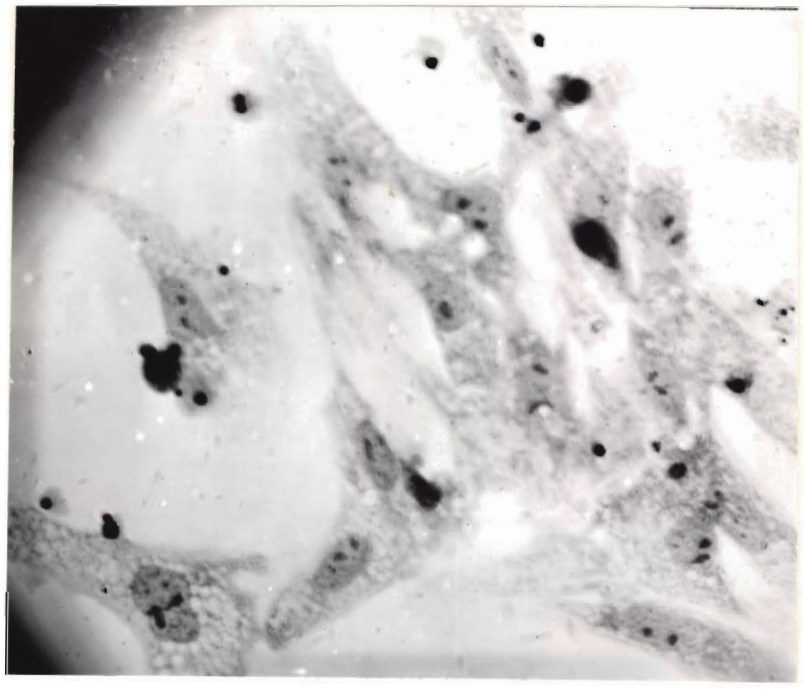

FIGURE # 9. The morphology of chick embryo cells infected with WEE virus variant L. P. # 7 and incubated at 25° C for 8 hours.

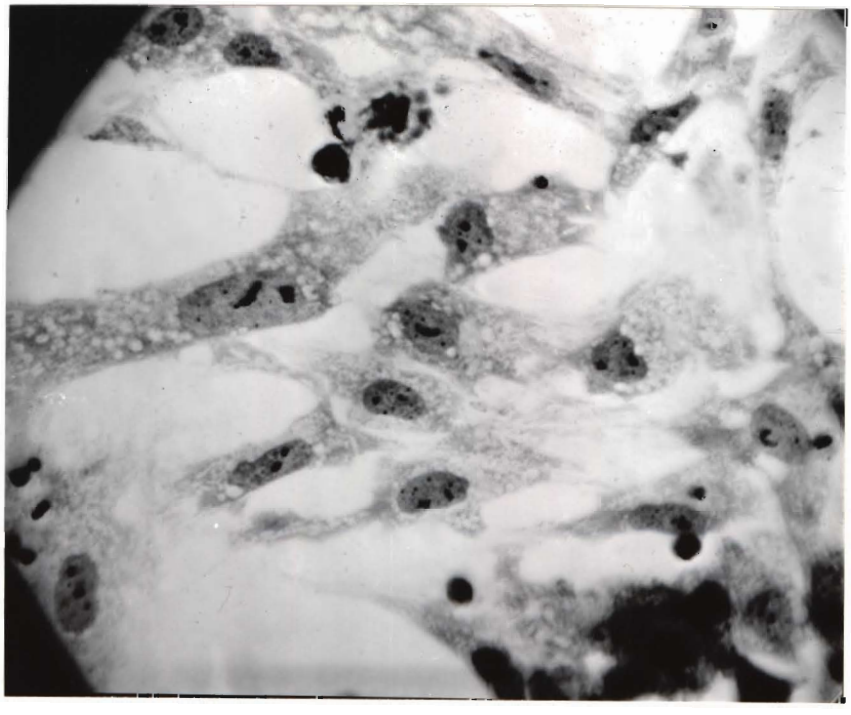

FIGURE # 10. Noninfected chick embryo cells after 24 hours of incubation at 25° C.

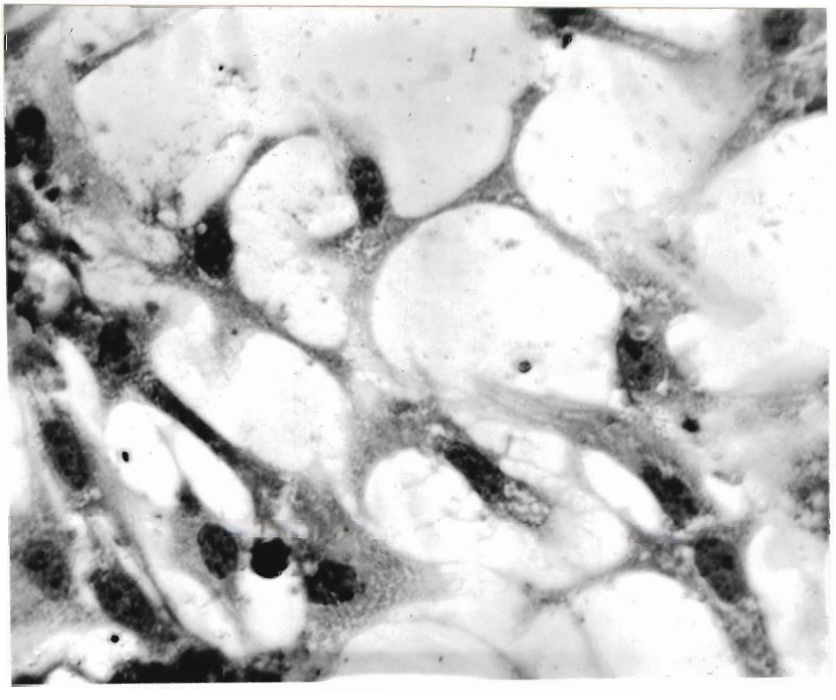

FIGURE # 11. S. P. # 6 infected chick embryo cells that have been incubated for 24 hours at 25° C.

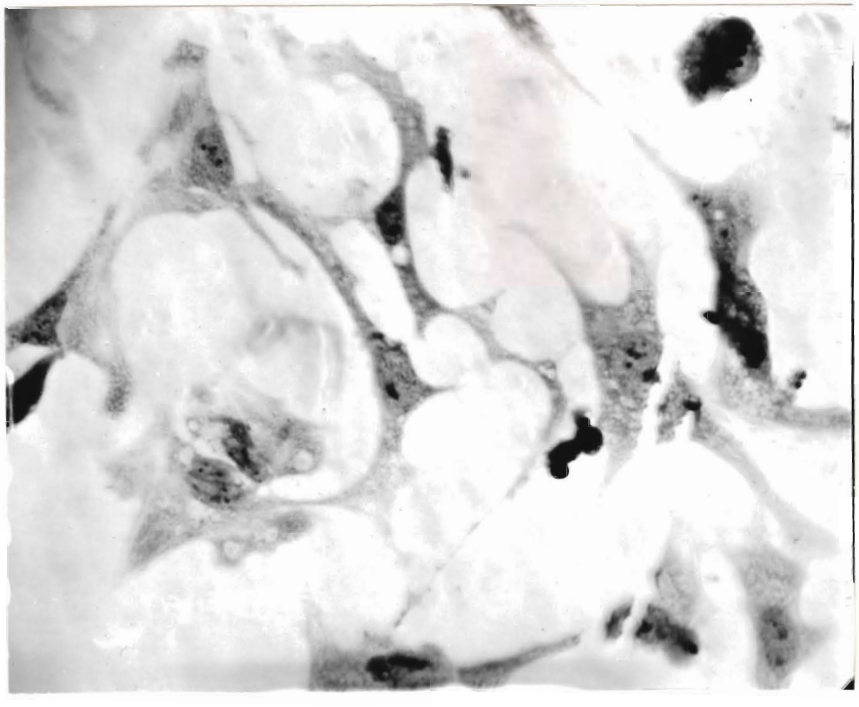

FIGURE # 12. A photomicrograph of chick embryo cells infected with L. P. # 7 after incubation at 25° C for 24 hours.

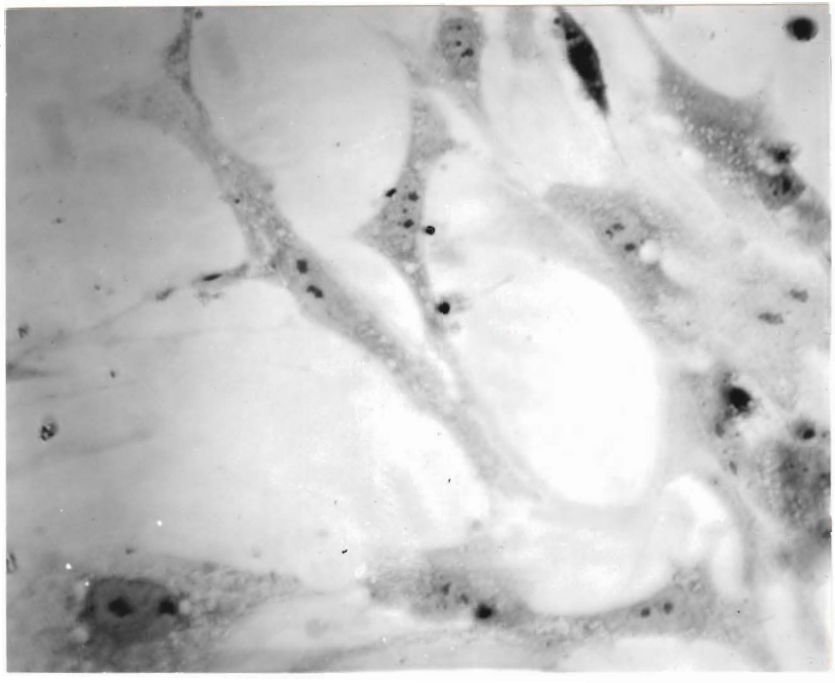

FIGURE # 13. The morphological appearance of noninfected chick embryo cells after 72 hours of incubation at 25° C.

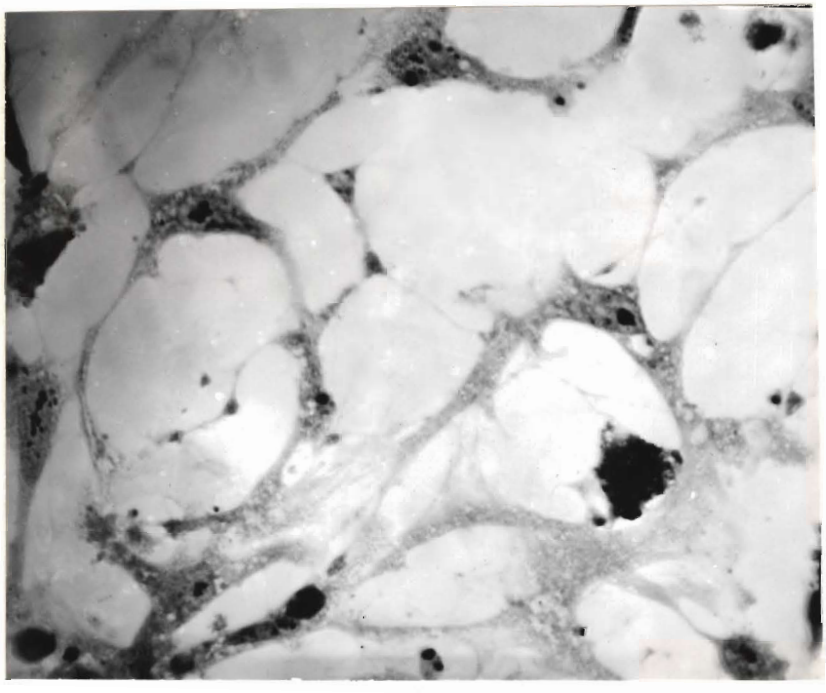

FIGURE # 14. Cytopathogenicity of S. P. # 6 infected chick embryo cells.

with extensive retraction of the cytoplasm, presence of large amounts of cellular debris and fewer numbers of cells attached to the glass. The nuclear membrane of many cells was broken at some points and the nuclear material, in some instances, had become indiscrete. FIGURE # 15 shows the appearance of L. P. # 7 infected cells after the same length of incubation. The cells appeared similar to those infected with S. P. # 6 except that the changes in the cells were more extensive and fewer cells remained attached to the glass.

2. WEE VIRUS INFECTED CELLS INCUBATED AT 37° C

a. Experimental.

The chick embryo monolayers that were infected with the two variants and incubated at 37° C exhibited basically the same morphological changes except that they developed over a shorter period of time.

FIGURE # 16, 17, and 18 present the appearance of the control, S. P. # 6 and L. P. # 7 infected cells incubated for 1 hour at 37° C. The cells appeared normal in every respect. However, after 8 hours of incubation cytopathogenicity could be detected. FIGURE # 21 shows L. P. # 7 infected cells 8 hours after incubation. The cells exhibited cytopathic changes with retraction of the cytoplasm and the appearance of cellular debris. The chick embryo cells infected with S. P. # 6 had not developed readily distinguishable cytopathogenicity by the same time (FIGURES # 19 and 20). Retraction of the cytoplasm

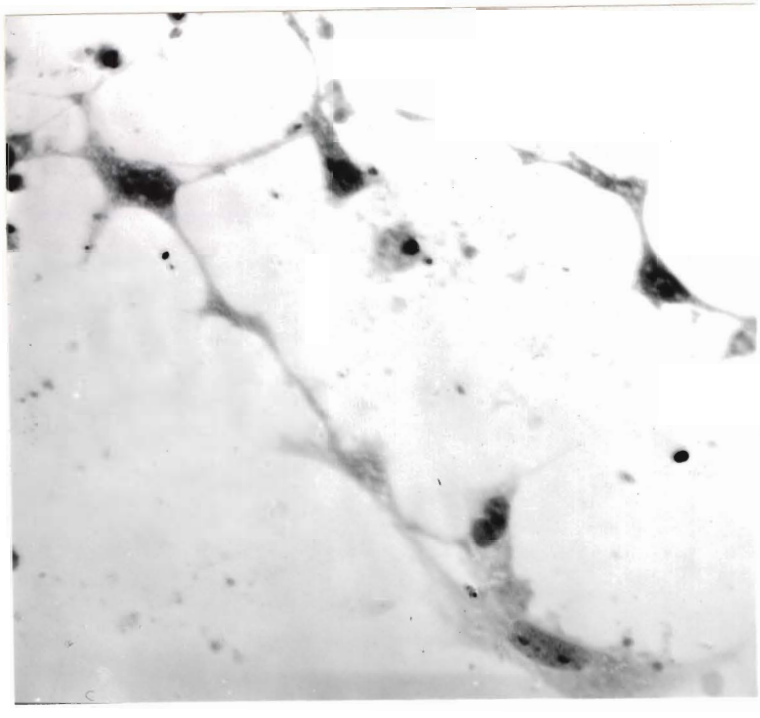

FIGURE # 15. Appearance of chick embryo cells that have been infected with L. P. # 7 and incubated 72 hours at 25° C.

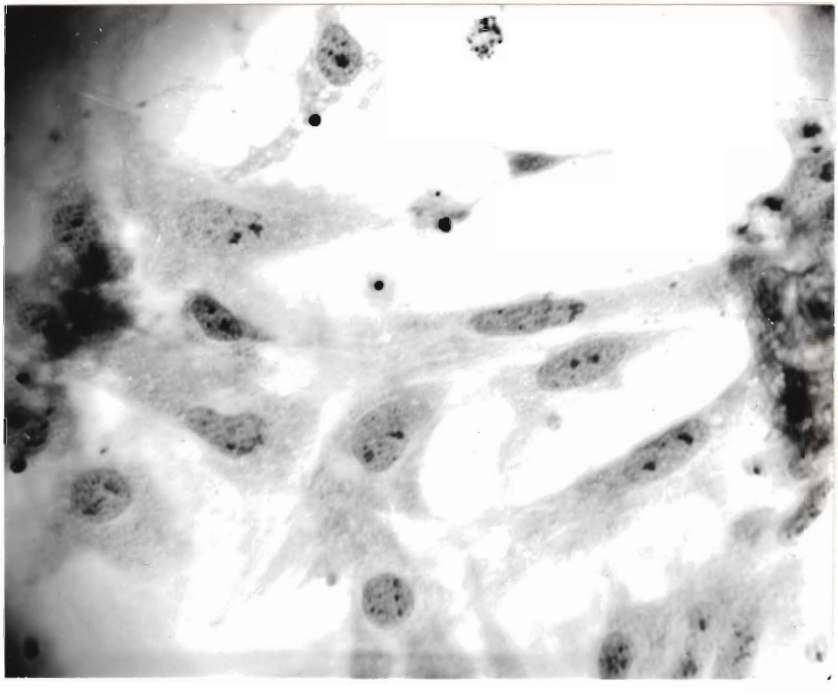

FIGURE # 16. Noninfected chick embryo cells after 1 hour of incubation at 37° C.

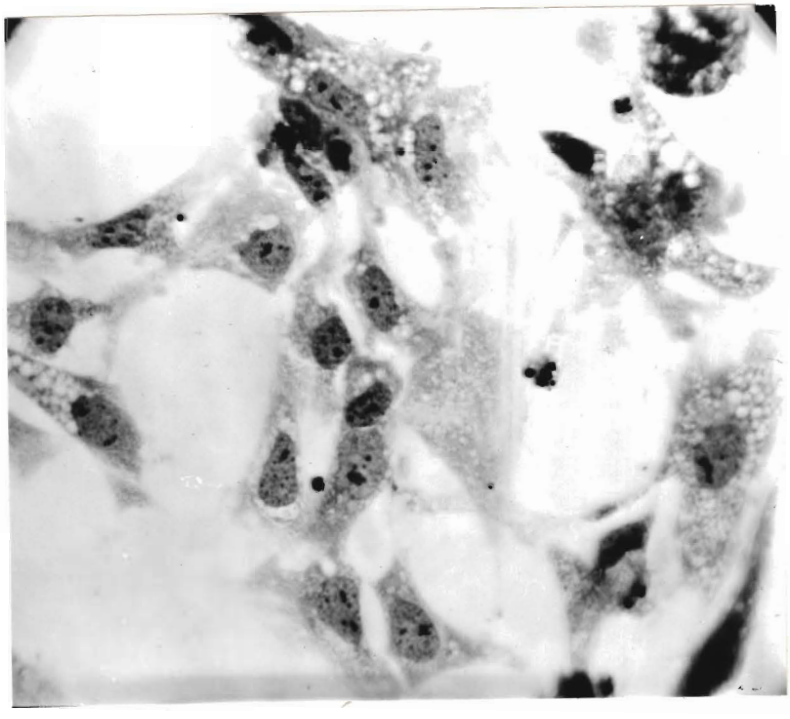

FIGURE # 17. Morphology of chick embryo cells that have been infected with WEE virus variant S. P. # 6 and incubated at 37° C for 1 hour.

FIGURE # 18. A photomicrograph of L. P. # 7 infected chick embryo cells after 1 hour of incubation at 37° C.

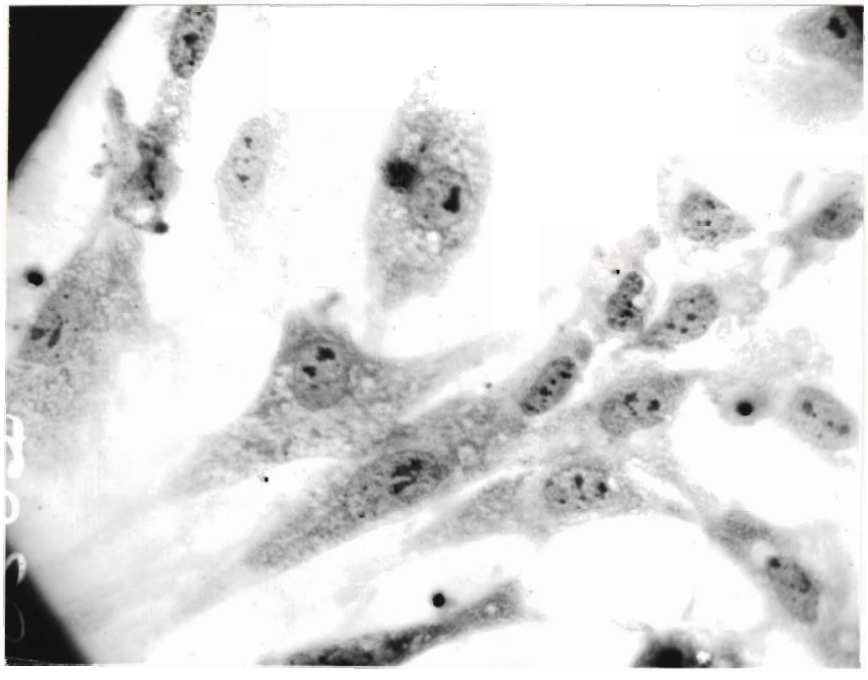

FIGURE # 19. Morphological appearance of noninfected chick embryo cells that have been incubated for 8 hours at 37° C.

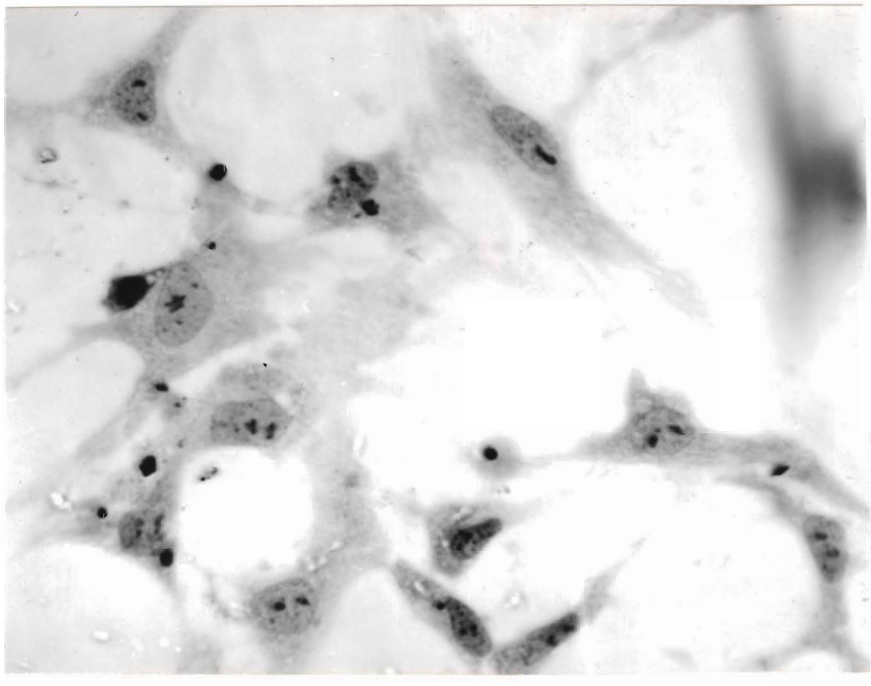

FIGURE # 20. Appearance of chick embryo cells infected with S. P. # 6 after 37° C incubation for 8 hours.

could be observed in a few cells but it was very difficult to distinguish these cells from the noninfected controls.

It is emphasized that the cells infected with L. P. # 7 develop cytopathic changes sooner than cells infected with the small plaque variant, also that the increase of PFU during the propagation of L. P. # 7 at 37° C was detected sooner than for S. P. # 6 (FIGURES # 2 and 4.) It should also be noted that cytopathogenicity of the cells developed just before the maximum titer was attained during the propagation of these two viruses.

FIGURES # 23 and 24 show the appearance of chick embryo cells infected with S. P. # 6 and L. P. # 7 after 24 hours of incubation at 37° C. Almost complete destruction of the cells had taken place and the number of cells remaining attached to the glass was decreased as compared to the noninfected control (FIGURE # 22).

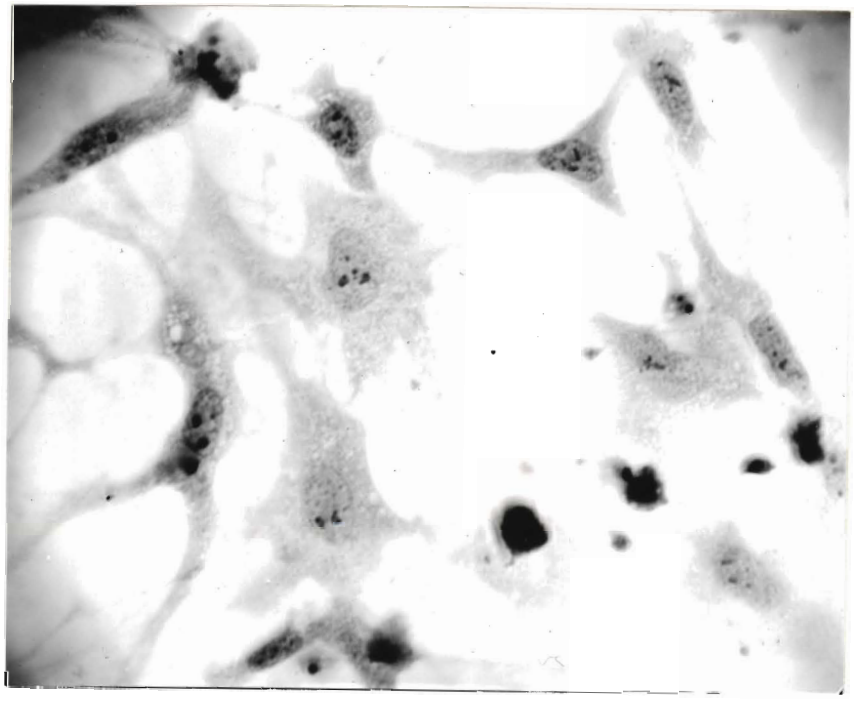

FIGURE # 21. Photomicrograph showing cytopathic changes of chick embryo cells infected with L. P. # 7 after 8 hours of incubation at 37° C.

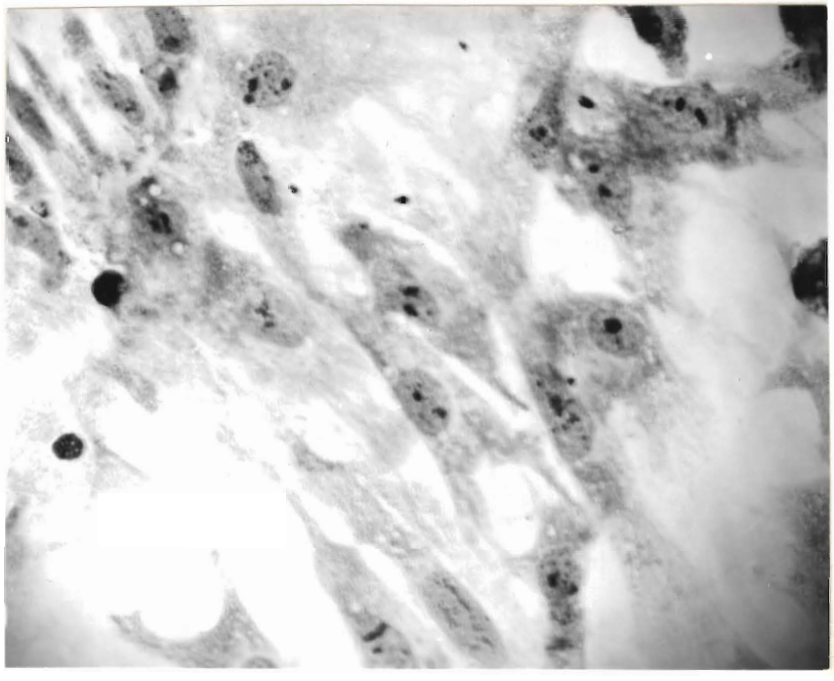

FIGURE # 22. Noninfected chick embryo cells 24 hours after incubation at 37° C.

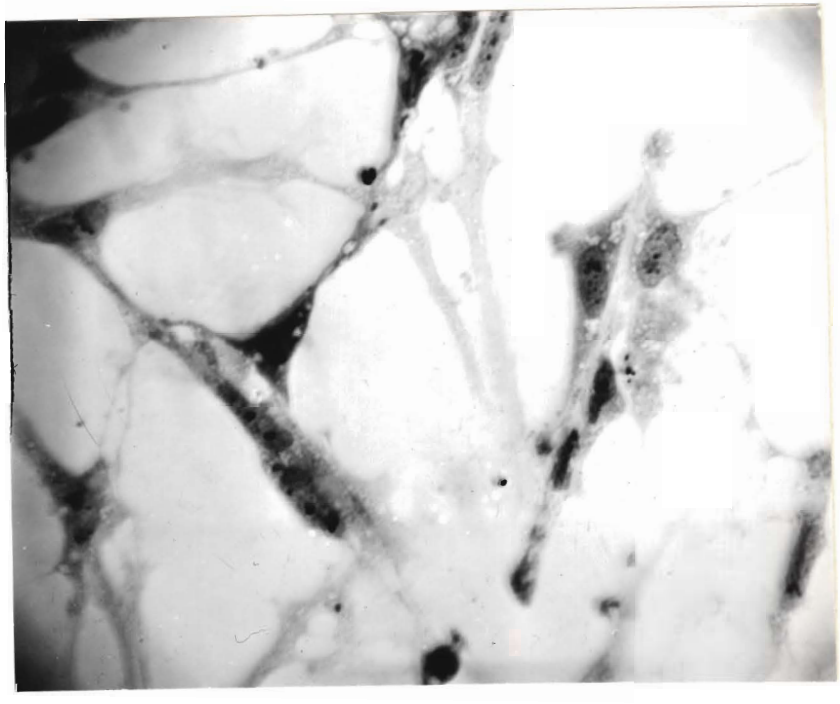

FIGURE # 23. Morphology of S.P. # 6 infected chick embryo cells that have been incubated at 37° C for 24 hours.

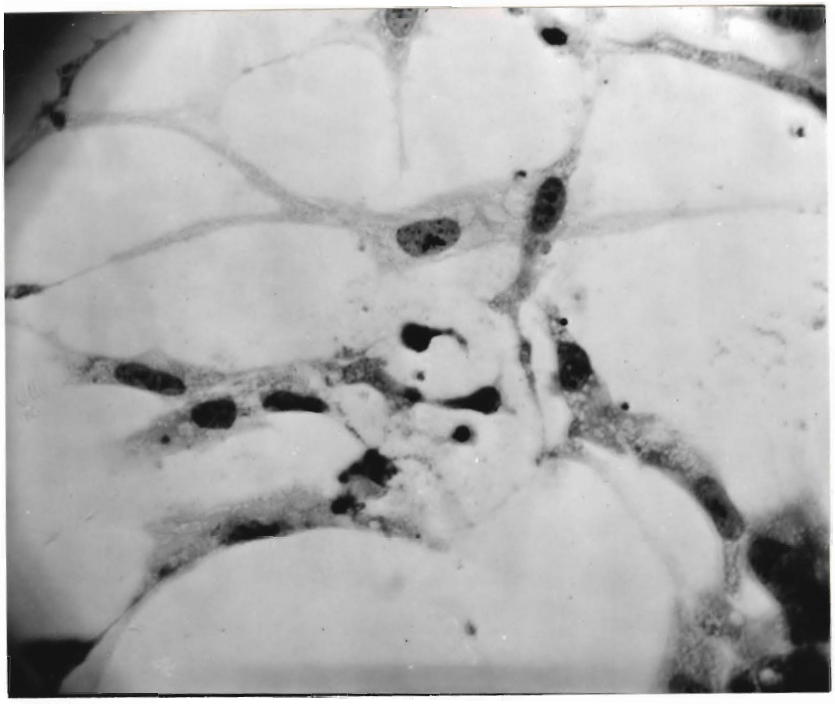

FIGURE # 24. Chick embryo cells that have been infected with L. P. # 7 and incubated at 37° C for 24 hours.

DISCUSSION

One of the main aspects of this work was concerned with comparative studies of the replication of two plaque variants, S. P. # 6 and L. P. # 7, of WEE virus at 25° and 37° C in chick embryo cells. The characteristics of growth of WEE virus have been studied previously at 37° C in chick embryo cells maintained as a monolayer and in suspension (46, 47). Of prime concern was the comparison of the effects of temperature on the rates of propagation of the two variants.

The existence of greater numbers of PFU of S. P. # 6 per ml in the extracellular fluid at time zero (FIGURES # 3, 5, and 6) was always noted in the course of this study except with the growth curve of S. P. # 6 and L. P. # 7 in monolayers at 37° C (FIGURE # 4). The chick embryo cells were infected with approximately the same number of plaque forming units, the length of the attachment period was the same and the infected cells were washed 2 times with similar volumes of cold growth medium. If the explanation for this observation was the elution of larger numbers of S. P. # 6 than L. P. # 7 from the infected cells after washing of the monolayers, it would suggest that S. P. # 6 does not attach as firmly to the chick embryo cells as does L. P. # 7 and hence can be eluted from the host cells more easily.

The length of the lag periods exhibited during the growth cycle of the variants was the same when the incubation temperature was

25° C. However, when virus infected cells were incubated at 37° C the lag periods were significantly different. From these data it may be suggested that the large plaque variant is more adapted to propagation at 37° C than is S. P. # 6, however, these same data also suggest that the small plaque variant is more adapted to propagation at 25° C. The length of the lag period, however, should not be the only factor considered as an indicator of the ability of a virus to propagate at a specific temperature. The maximum titer obtained, the number of PFU released per cell along with the length of time required for each to be attained and the rate of increase of PFU released into the extracellular fluid should also be considered.

The rate of increase of PFU of L. P. # 7 in the extracellular fluid was always greater than or as great as the increase for S. P. # 6 whether the incubation temperature was 25° or 37° C. The length of time required for the maximum number of PFU present to be released was dependent upon the temperature of incubation and the cell culture system used. For example, after about a 10 hour period of incubation at 37° C a maximum titer was reached whether the cells infected with the two variants were in suspension or as a monolayer. However, when similar virus infected cells were incubated at 25° C the length of incubation to attain a maximum titer from cells in suspension was shorter than when cells were maintained as a monolayer. The large plaque variant attained a higher titer in the extracellular fluid than

did S. P. # 6 except when infected cells were maintained in suspension and incubated at 25° C.

The studies concerning the maximum number of PFU released per cell demonstrated that the yield per cell infected with L. P. # 7 drops 10 fold with the decrease in temperature of incubation from 37° to 25° C. In contrast the number of PFU of S. P. # 6 released per cell when incubated at 25° C was about the same as at 37° C.

Experiments were conducted to compare the development of cytopathic changes in S. P. # 6 and L. P. # 7 infected chick embryo cells incubated at 25° and 37° C. Cytopathogenicity did not develop in infected cells incubated at 25° C until after 24 hours of incubation. The cytopathic changes were more evident in the cells infected with L. P. # 7 and could be correlated with the more rapid increase in extracellular virus of the large plaque variant as opposed to S. P. # 6. Similar results were obtained with incubation at 37° C. Earlier cytopathic changes at 37° C in L. P. # 7 infected cells were also correlated with the shorter lag period in the growth cycle of the large plaque variant. It is of interest to note that the noninfected cells incubated for more than 8 hours at 25° C were not normal in appearance, as was evidenced by the morphological appearance of the cells. The cell cytoplasm was retracted and also showed less vacuolation.

At the higher temperature cytopathogenicity of the infected cells was first evident at 8 hours after incubation and it should be emphasized that in comparing the appearance of cytopathogenicity of infected cells

incubated at 37° and 25° C several factors must be considered. Three of these factors are: (a) the time difference in the appearance of cytopathogenicity at the two temperatures, (b) correlation of the growth cycle at the different temperatures with the cytopathic changes of the infected cells, and (c) the effect the lowering of the temperature has upon the morphological appearance and energy yielding processes of the cells should all be considered.

As observed there was a 16 hour time difference in the appearance of cytopathic changes at 37° C as opposed to 25° C. However, if a comparison of the growth cycle and development of cytopathic changes is made, it was noted that cytopathogenicity developed earlier during the growth cycle when the infected cells were incubated at 25° C. Cells incubated at 25° C had observable changes before the cells had released 1 PFU per cell into the extracellular fluid while cytopathogenicity did not develop in cells incubated at 37° C until almost a maximum release of virus per cell had occurred. However it is emphasized that noninfected cells incubated at 25° C for 24 hours did not appear as normal as cells incubated at 37° C. Morgan et al. (70) have suggested that the development and ultimate release of WEE virus from infected cells may occur by several mechanisms and the release of particles from a cell by any of these means may result in the loss of cellular material or change in cell membrane permeability and cell cytopathic changes would develop.

SUMMARY

The propagation of WEE virus variants L. P. # 7 and S. P. # 6 infected chick embryo cells maintained as monolayer and in suspension was studied at 25° and 37° C. The results may be summarized as follows:

1. The lag period of the growth cycle for S. P. # 6 and L. P. # 7 was found to be 10 hours when the infected cells were maintained as a monolayer or in suspension and incubated at 25° C. The lag period for S. P. # 6 infected cells in suspension and as a monolayer incubated at 37° C was found to be 2 hours while that for L. P. # 7 was 1 1/2 hours.

2. The maximum number of PFU of both variants present in the extracellular fluid was reached after 72 hours of incubation of infected monolayers at 25° C. With S. P. # 6 infected cells in suspension the maximum titer of S. P. # 6 PFU was attained after 48 hours of incubation at 25° C while with L. P. # 7 infected cells the maximum PFU was reached after only 36 hours of incubation at 25° C. S. P. # 6 infected cells in suspension, however, released a larger number of PFU than did L. P. # 7 infected cells. The reverse was true with cells maintained as a monolayer.

3. The maximum number of PFU released into the extracellular fluid by S. P. # 6 and L. P. # 7 infected cells in suspension or maintained as a monolayer occurred after 10 hours of incubation at 37° C. The highest titer was produced by cells infected with the large plaque variant.

4. On the basis of infective center assay and counts of replicate noninfected monolayers the number of PFU released per infected cell was determined.

An experiment was designed to study the cytopathic changes that develop in WEE virus infected chick embryo cells incubated at 25° and 37° C. The results of this experiment may be summarized as follows:

1. Cytopathogenicity was first observed in L. P. # 7 infected cells after 8 hours of incubation at 37° C. S. P. # 6 infected cells did not show definite signs of cytopathic changes until after 10 hours of incubation at 37° C.

2. Infected cells incubated at 25° C developed observable cytopathogenicity after 24 hours of incubation.

3. Comparisons were made with the development of cytopathogenicity and the propagation of the two variants at the two experimental temperatures.

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THE EFFECT OF TEMPERATURE ON THE INFECTIVITY AND PROPAGATION OF WESTERN EQUINE ENCEPHALITIS VIRUS

by

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On the basis of contemporary epidemiological and epizoological information the range of environmental temperatures that the arthropod viruses are exposed to is obviously considerable. Among the known natural hosts and vectors of the virus of Western equine encephalitis (WEE), a broad spectrum of normal body temperatures is evident. Studies were undertaken to provide some information on the effect of propagating WEE virus at varied temperatures. Emphasis was placed upon studies of the rate of propagation and cytopathogenicity of WEE virus as a function of temperature.

The propagation of 2 plaque variants of WEE virus, a large plaque L. P. # 7 and a small plaque S. P. # 6, was studied at 25° and 37° C in infected chick embryo cells maintained as a monolayer and in suspension. The lag period of the growth cycle for S. P. # 6 and L. P. # 7 was found to be 10 hours when the infected cells were maintained as a monolayer or in suspension and incubated at 25° C. The lag period for S. P. # 6 infected cells in suspension and as a monolayer incubated at 37° C was found to be 2 hours while that for L. P. # 7 was 1 1/2 hours. The maximum number of plaque forming units of both variants present in the extracellular fluid was reached after 72 hours of incubation of infected monolayers at 25° C with L. P. # 7 attaining a higher titer in the extracellular fluid than did S. P. # 6. When the infected cells were maintained in suspension at 25° C the maximum titer was reached after 48 hours with S. P. # 6 and after 36 hours with L. P. # 7, however, the titer of S. P. # 6 was greater than for the large plaque variant.

Incubation of similar systems at 37° C produced results that showed that a maximum number of PFU in the extracellular fluid was attained after 10 hours of incubation with the cells in suspension or as a monolayer. The highest titer was produced by cells infected with the large plaque variant. On the basis of infective center assays and counts of replicate noninfected monolayers the number of PFU released per infected cell was determined.

An experiment was designed to study the cytopathic changes that develop in WEE virus infected chick embryo cells as a monolayer incubated at 25° and 37° C. Cytopathogenicity of the infected cells was first observed after 8 hours of incubation at 37° C with cells infected with L. P. # 7 developing changes sooner than did cells infected with the small plaque variant. Infected cells incubated at 25° C showed cytopathic changes after 24 hours of incubation. Comparisons were made with the development of cytopathogenicity and the propagation of the 2 variants at the experimental temperatures. Cytopathogenicity developed in infected cells incubated at 25° C earlier in the growth cycle than at 37° C.